

Levels of Calcium in the Skin of Some Amphibians and Possible Evolutionary Implications

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ABSTRACT—Levels of calcium (Ca) in the skin were determined in several species of anuran and urodele obtained from various environments. One species of apodan was also examined as a reference, which is completely aquatic. We confirmed that, in general, amphibians contain large amounts of Ca in the skin. However, it was clear that Ca levels in the skin are greater in anurans than in urodeles, although even among anurans there were some variations. Furthermore, it was demonstrated that, in anurans, levels of Ca are higher in the dorsal skin than in the ventral skin in species adapted to semi-terrestrial and semi-aquatic environments. This was not the case for the species that inhabit an aquatic environment.

INTRODUCTION

In vertebrates, Ca is the most important constituent of skeletal bones. Amphibians were the first vertebrates that successfully adapted to semi-terrestrial environments. Modern amphibians consist of three groups: the anurans (frogs and toads); urodeles (newts and salamanders); and the apodans (legless newts). It has been reported that, in some species of anurans, the skin, and especially the dorsal skin, contains a large amount of Ca [1, 4–6]. It is not clear, however, whether or not this is a general phenomenon in anurans, nor is it clear whether such is also the case in urodeles and apodans. Therefore, in the present study, levels of Ca in the skin of several species in the three groups of amphibians, obtained from a variety of environments, were determined. The results are discussed from some aspects of the mode of living in amphibians.

MATERIALS AND METHODS

Five species each of anurans and urodeles were examined. In addition, one species of apodan was included as a reference. Those are listed in Table 1.

In the present study, at first it examined whether or not levels of Ca in the skin of anurans and urodeles are actually higher than in other soft tissues. After animals had been anesthetized with 0.03% ethyl 3-aminobenzoate methanesulfonate (Nakarai Tesque, Inc), about 20 kinds of tissue were dissected out from various parts of the body and immediately weighed. In *Hymenochirus* sp., however, only two kinds of tissue were taken out, and in *Typhloreutes compressicauda*, five kinds of tissue. These tissues were then solubilized in a mixture of nitric acid and perchloric acid (11:2, v/v). The resultant solutions were diluted appropriately with distilled water, and total Ca concentrations were determined by atomic absorption spectrophotometry. Levels of Ca were calculated from the extent of dilution and the final concentration of Ca in each solution.

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TABLE 1. Amphibians used in the present study

Species	Number	Sex	Body weight (g, average)
Anurans			
<i>Hyla japonica</i>	10	m=8, f=2	2.2
<i>Rana nigromaculata</i>	9	m=5, f=4	14.9
<i>Rana japonica</i>	13	m=5, f=8	10.8
<i>Rhacophorus schlegelii</i>	6	m=3, f=3	4.6
* <i>Hymenochirus</i> sp.	4	—	8.6
Urodeles			
<i>Cynops pyrrhogaster</i>	10	m=5, f=5	5.5
<i>Onychodactylus japonicus</i>	10	m=5, f=5	7.3
<i>Hynobius nigrescens</i>	11	m=11	17.2
<i>Hynobius lichenatus</i>	6	m=6	5.0
<i>Ambystoma mexicanum</i>	5	m=3, f=2	117.1
Apodan			
<i>Typhlonectes compressicauda</i>	2	—	30.0

* Species was not specified. m, male; f, female.

The dorsal and ventral skins were fixed with a neutralized solution of 5% formalin to determine the sites of Ca in the skin. Specimens were treated histochemically with von Kossa's stain for light-microscopic observations. A portion of each sample of skin was also analyzed by an X-ray microanalyzer (JCXA-733 with Kevex 7045 energy dispersive X-ray spectroscopy) to confirm the exact location of Ca.

RESULTS

Levels of Ca in skin and in other tissues

Levels of Ca in various tissues from five species of anuran are summarized in Table 2. In Figure 1, levels of Ca in tissues from *Rhacophorus schlegelii* are shown in a histogram as an example. The average level of Ca in the skin was clearly higher than that in other tissues except hard tissues. The value of Ca in the skin was 17 times higher than the average value for the other soft tissues (Table 2). Similar data were obtained in other species (Table 2), level of Ca in the skin being 16 times higher in *Hyla japonica*, 58 times higher in *Rana japonica*, and 109 times higher in *Rana nigromaculata* than the average value of other soft tissues.

In most of the anurans examined, levels of Ca in

the dorsal skin were higher in the anterior portion than in other portions (Table 2). Furthermore, in *Rh. schlegelii*, the level of Ca in the anterior portion of the dorsal skin was 3.9 times higher than that in the ventral skin (Fig. 2). In other species, as shown in Figure 2, levels were 2.0 times higher in *R. nigromaculata* and 14.6 times higher in *H. japonica*, while in *Hymenochirus* sp. and *R. japonica* dorsal and ventral levels were almost the same.

The level of Ca in the dorsal skin was highest in *R. nigromaculata* (Fig. 2). There were no conspicuous differences in the level of Ca in the dorsal skin between *R. japonica* and *H. japonica*. Levels of Ca in the dorsal skin of *Rh. schlegelii* and *Hymenochirus* sp. were low compared to those in other anurans.

In Table 3, levels of Ca in the skins and other tissues of five species of urodele and one species of apodan are summarized. Levels in the skin of urodeles were extremely low when compared to those in the skin of anurans (Fig. 2). In Figure 3, the levels of Ca in tissues of *Cynops pyrrhogaster* are shown in a histogram as an example. In urodeles, levels of Ca in the skin were high after levels in gall bladder and hard tissues (Table 3), although the difference between the skin and other soft tissues was small when compared to that

TABLE 2. Levels of Ca ($\mu\text{g/g}$ fresh tissue) in various tissues and organs of five species of anuran

Tissue/Organ	Species HJ (n=10)	RN (n=9)	RJ (n=13)	RhS (n=6)	HY (n=4)
Ventral skin (a)	190.3	3250.4	3295.7	516.9	—
Ventral skin (m)	130.8	3402.2	1929.6	115.4	961.3
Ventral skin (p)	152.2	2917.6	807.5	184.2	—
Dorsal skin (a)	2778.0	6447.1	3550.4	2034.5	—
Dorsal skin (m)	2295.2	5597.3	2975.6	1612.6	1111.5
Dorsal skin (p)	1178.3	3693.8	2308.0	1457.0	—
Stomach	64.9	57.7	83.3	56.7	—
Duodenum	76.1	46.8	135.6	89.8	—
Intestine	99.7	58.7	81.6	83.3	—
Rectum	140.5	74.5	71.3	95.7	—
Liver	32.7	42.6	38.0	17.2	—
Gall bladder	285.5	240.4	382.8	516.9	—
Pancreas	126.2	86.8	80.5	153.2	—
Spleen	47.9	41.6	83.5	48.0	—
Lung	61.6	68.9	81.9	48.5	—
Kidney	51.0	50.4	52.8	33.2	—
Heart	52.6	60.9	56.5	54.9	—
Aorta	304.0	150.6	135.6	7.3	—
Testis	42.7	53.8	21.5	18.4	—
Ovary	48.7	30.7	49.1	6.5	—
Fat body	137.0	15.1	40.4	88.4	—
Muscle	111.8	76.7	107.3	102.8	—
Nerve	112.3	124.7	54.1	123.5	—
Lime sac	29167.4	146278.5	145188.3	250479.2	—
Humerus	162582.7	117559.5	348008.9	81954.3	—
Femur	166262.7	173162.7	221450.1	155195.3	—
Vertebra	151041.0	143578.4	160614.3	108303.9	—
Cartilage	14380.5	23135.7	17765.1	14562.6	—

Numbers in parentheses indicate numbers of animals used.

HJ, *Hyla japonica*; RN, *Rana nigromaculata*; RJ, *Rana japonica*; RhS, *Rhacophorus schlegelii*; HY, *Hymenochirus* sp.

(a), anterior; (m), middle; (p), posterior.

observed in anurans (Table 2). The level of Ca in the skin was 2.9 times higher in the newt than the average value of other soft tissues. In other three species of urodeles (*Hynobius lichenatus*, *Hynobius nigrescens* and *Ambystoma mexicanum*), levels of Ca in the skin were 1.5–1.6 times higher than the average values in other soft tissues. In *Onychodactylus japonicus*, there were no differences in levels of Ca between the skin and other soft tissues. In urodeles, in both the dorsal skin and the ventral skin, there were no clear differ-

ences in levels of Ca among different regions (Table 3). Furthermore, although levels of Ca in the anterior portion of the dorsal skin were higher than those in the ventral skin, the differences were smaller (1.1–1.7 times) (Fig. 2) as compared to those in anurans (1.1–14.6 times).

The levels of Ca in the skin was highest in *C. pyrrhogaster* (Fig. 2), and next highest in *A. mexicanum*. There were no conspicuous differences among the other three species, *H. lichenatus*, *H. nigrescens* and *O. japonicus*.

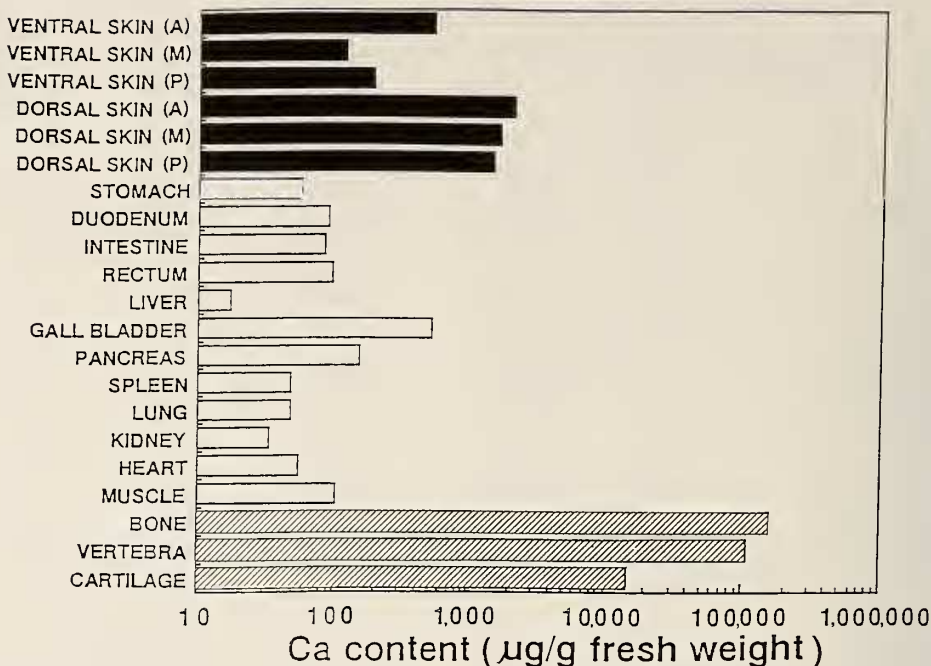


FIG. 1. Levels of Ca in various tissues of an anuran, *Rhacophorus schlegelii*. The values for the skin are shown as black columns. Values for other soft tissues and hard tissues are shown as white columns and striped columns, respectively. Levels of Ca contents are indicated on a logarithmic scale. A, Anterior; M, Middle; P, Posterior.

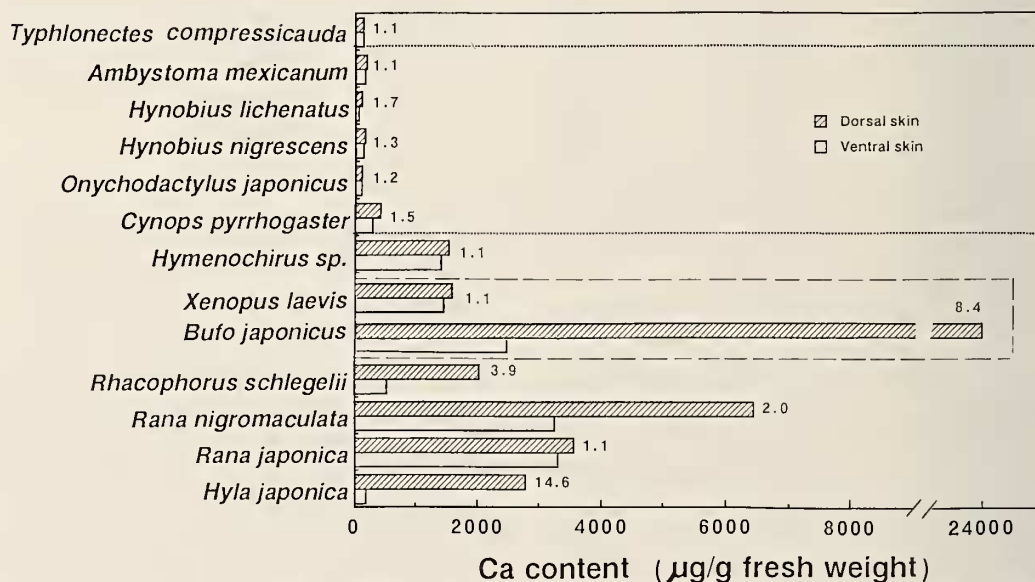


FIG. 2. Levels of Ca in the dorsal skin (striped columns) and the ventral skin (white columns) of various species of amphibian. Numbers to the right of columns are the ratios of levels of Ca in the dorsal skin to those in the ventral skin. In the Figure, results obtained for *Bufo japonicus* [5] and *Xenopus laevis* (Oguro, unpublished data) are added (inside of broken line).

TABLE 3. Levels of Ca ($\mu\text{g/g}$ fresh tissue) in various tissues and organs of five species of urodele and one species of apodan

Tissue/Organ	Species (n=10)	OJ (n=10)	HN (n=11)	HL (n=6)	AM (n=5)	TC (n=2)
Ventral skin (a)	275.2	95.6	117.2	64.5	163.1	139.9
Ventral skin (m)	224.3	112.9	112.5	71.4	186.8	157.7
Ventral skin (p)	286.6	138.1	94.8	77.3	175.5	96.0
Dorsal skin (a)	416.7	112.7	149.8	111.0	179.1	143.6
Dorsal skin (m)	416.2	108.9	154.3	116.3	204.2	168.1
Dorsal skin (p)	373.4	115.4	157.0	136.0	305.7	161.3
Stomach	107.7	72.7	79.2	60.0	174.8	—
Duodenum	87.4	63.6	66.3	50.4	137.0	—
Intestine	81.3	69.7	80.3	50.6	107.7	—
Rectum	92.5	79.3	75.5	64.9	196.5	—
Liver	24.6	21.0	42.6	23.0	67.6	—
Gall bladder	428.7	485.6	337.6	227.6	—	—
Pancreas	116.9	89.4	—	—	133.7	—
Spleen	34.6	21.5	48.6	24.4	64.8	—
Lung	98.1	—	60.6	52.6	158.0	—
Kidney	116.9	90.4	59.3	57.8	237.1	—
Heart	101.7	103.0	—	60.7	103.9	—
Testis	14.1	47.0	23.2	20.2	140.5	—
Spermiduct	58.8	100.9	23.4	27.9	—	—
Ovary	155.4	34.7	—	—	71.4	—
Oviduct	186.8	382.7	—	—	—	—
Muscle	113.0	104.9	64.1	136.1	180.5	—
Bone	212454.9	128556.8	171819.3	205081.8	357352.5	—
Vertebra	132891.4	131090.1	81711.5	128366.3	264050.8	—
Cartilage	20897.1	10453.0	29572.9	17267.0	815.5	—

Numbers in parentheses indicate numbers of animals used.

CP, *Cynops pyrrhogaster*; OJ, *Onychodactylus japonicus*; HN, *Hynobius nigrescens*; HL, *Hynobius lichenatus*; AM, *Ambystoma mexicanum*; TC, *Typhlonectes compressicauda*.

(a), anterior; (m), middle; (p), posterior.

In the apodan, *Typhlonectes compressicauda*, only the level of Ca in the skin was examined (Table 3, Fig. 2). In this species, there were no clear differences in levels between the dorsal skin and the ventral skin, as indicated by an examination of only two individuals.

Histochemical observations of skin after von Kossa staining

In the dorsal skin of anurans (*Hymenochirus* sp. was not examined), cells of the spongy layer that covers the compact layer, in the dermis, were positive for von Kossa staining. Cytoplasm of

those cells was tinged with brown, which demonstrates the presence of Ca (Fig. 4). In the ventral skin, however, the cells with brown cytoplasm did not constitute a continuous spongy layer.

In all species of urodele examined, there were no clear layers of cells that were positive for von Kossa staining in either the dorsal skin or the ventral skin.

Analysis of skin with an X-ray microanalyzer

In anurans, only the brown parts of the cytoplasm, as stained by von Kossa's method, gave sharp peaks upon microanalysis, indicating the

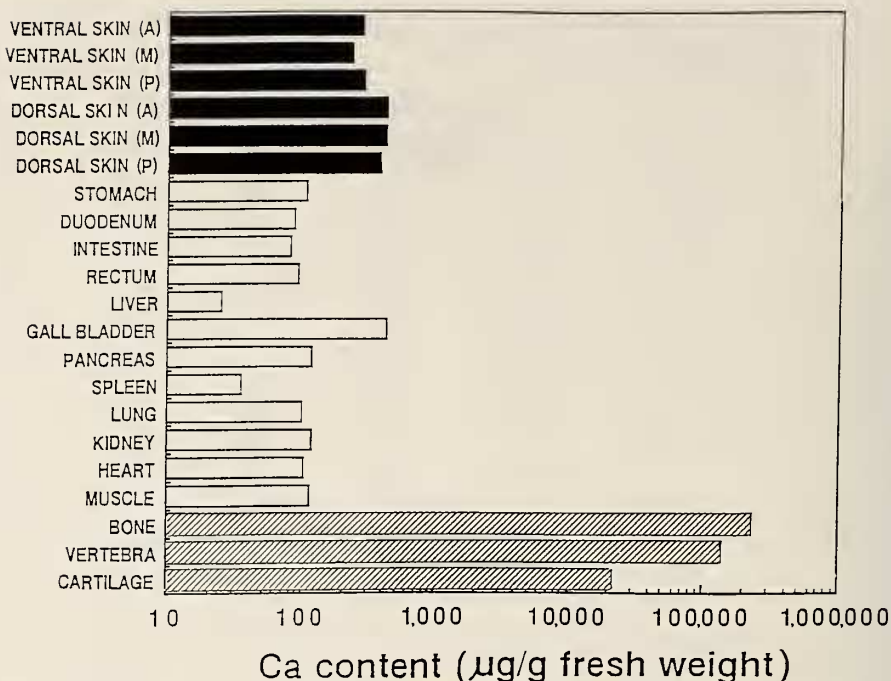


FIG. 3. Levels of Ca in various tissues of a urodelan species, *Cynops pyrrhogaster*. The values for the skin are shown as black columns. Values for other soft tissues and hard tissues are shown as white columns and striped columns, respectively. Levels of Ca are indicated on a logarithmic scale. A, Anterior; M, Middle; P, Posterior.

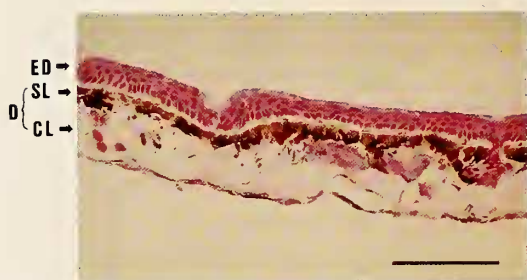


FIG. 4. Cross section of the dorsal skin of *Rhacophorus schlegelii*, stained by von Kossa's method. The skin is composed of the epidermis (ED) and the dermis (D) which is made up of two layers, the spongy layer (SL) and the compact layer (CL). Ca is stained brown in the cytoplasm of the spongy layer cells. Black areas are melanin granules. Bar represents 100 μm .

presence of Ca and phosphorus. In Figure 5A, the result for *Rh. schlegelii* is shown as a typical example.

In urodeles, small peaks due to Ca and phosphorus were observed only in the dorsal skin of *C.*

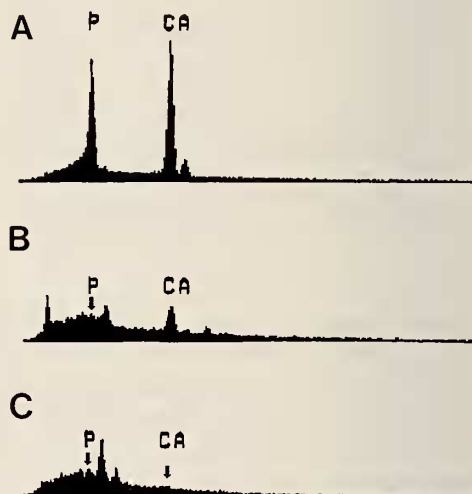


FIG. 5. Point-analysis at the spongy layer with an X-ray microanalyzer. A: *Rhacophorus schlegelii*; B: *Cynops pyrrhogaster*; C: *Onychodactylus japonicus*. Detection of Ca and phosphorus was performed for 60 sec within energy range 0–40 K electron volt (horizontal axis). Those spectra were obtained at 10.24 K electron volt. The sensitivity of vertical axis was set up 8192 (A) or 4096 (B, C) count values.

pyrrhogaster (Fig. 5B). In other species of urodeles examined, no clear peaks were detected from either the dorsal skin or the ventral skin (Fig. 5C). In the case of *A. mexicanum* and *T. compressicauda*, no X-ray analysis was performed.

DISCUSSION

From the present results, it is clear that, in four genera of anurans, the skin contains more Ca than other soft tissues, but less than the hard tissues. Also in four genera of urodeles, levels of Ca in the skin were higher compared to those in other soft tissues except gall bladder. Therefore, it seems reasonable to conclude that amphibians generally have high levels of Ca in the skin. It has been reported that in mammals, Ca is excreted from liver into bile [8]. The physiological importance of the accumulation of Ca in the skin has so far been discussed only in anurans. It has been proposed that Ca protects against desiccation of the skin [4], that the skin stores Ca [1, 9] and that absorption of Ca occurs through the skin [7]. The present data do not exclude these possibilities.

Taylor *et al* [6] reported that Ca in the skin must be bound to phosphorus. The results from an X-ray microanalysis in the present study suggest that this supposition is reasonable. Furthermore, they reported that, in *Rana catesbeiana*, Ca is present as crystalline granules that are distributed in the intercellular space, covering the compact layer in the dermis. A similar distribution of Ca has been demonstrated in *Rana arvalis* and *Rana ridibunda* [4]. In the present study, by contrast, in *H. japonica*, *R. nigromaculata*, *R. japonica* and *Rh. schlegelii*, Ca was detected in the cytoplasm of cells of the spongy layer that covers the compact layer. The reason for this discrepancy is not clear at the present.

We found a clear difference in the level of Ca in the skin between anurans and urodeles. In anurans, the level of Ca was very higher than in urodeles. In Figure 2, level of Ca in the skins examined in the present study and data from *Bufo japonicus* and *Xenopus laevis*, which were obtained previously ([5] and Oguro, unpublished data), are shown together. Moreover, in most species of anuran, levels of Ca were higher in the

dorsal skin than the ventral skin. Therefore, this conclusion, suggested originally for *B. japonicus* [5], can be applied to most anurans. In *H. japonica* and *B. japonicus*, ratio of the level of Ca in the dorsal skin to that in the ventral skin was very high, being 14.6 and 8.4, respectively. In *Rh. schlegelii*, *R. nigromaculata* and *R. japonica*, ratios were 3.9, 2.0 and 1.1, in that order. In *X. laevis* and *Hymenochirus* sp. the ratio was 1.1 in both cases. By contrast, in urodeles, there were no major differences in the levels of Ca between the dorsal skin and the ventral skin. The ratios were 1.1–1.7.

It is well known that anurans that belong to Bufonidae and Hylidae have adapted to terrestrial life to a certain degree. With respect to classification of anurans, it has been indicated that Hylidae originated from some ancestor of Bufonidae [3]. Frogs of Ranidae and Rhacophoridae inhabit semi-aquatic and semi-terrestrial environments, and the latter group is phylogenetically descended from the former group [3]. Two species, *X. laevis* and *Hymenochirus* sp. are completely aquatic. These species are closely related in terms of classification. The urodeles examined in the present study were semi-terrestrial (*C. pyrrhogaster*, *O. japonicus* and two species of Hynobiidae) or aquatic (*A. mexicanum*). The one species of apodan was completely aquatic. Thus, it seems that, broadly speaking, species that inhabit similar environments and are phylogenetically closely-related have similar ratios for the levels of Ca in their dorsal and ventral skins.

As regard to the mode of locomotion, anurans belonging to Bufonidae and Hylidae usually do not swim but crawl. When they crawl, they advance using only their legs, without turning or twisting their bodies. When frogs of Ranidae swim, they never turn or twist their bodies. The backbone must be fixed for kicking water with the hind limbs. By contrast, urodeles advance by turning and twisting their bodies, in addition to using their legs. The body of the aquatic apodan used in the present study is so flexible that it can coil itself. Therefore, styles of locomotion may be related to the differences between anurans and urodeles in the levels of Ca in the skin. In anurans, the anterior portion of the dorsal skin contains more

Ca than other parts. In this portion of the frog body, movement seems to be least extensive. In urodeles, there were no differences in the levels of Ca among the different regions of the skin.

The ancient amphibians had an outer integument that resembled armor, which must have contained a large amount of Ca [2]. Therefore, it may be erroneous to suppose that amphibians adapting to a terrestrial environment accumulated Ca in their skin during evolution. Changes in levels of Ca in the skin might not represent a history of accumulation of Ca but of reductions in levels of Ca. It is of interest that *B. japonicus*, which is regarded as a primitive species, had the highest levels of Ca in the skin among amphibians examined. There is a possibility that rates of reduction of levels of Ca in the skin may differ from species to species or even from the dorsal skin to the ventral skin. In anurans, the ventral skin may have to be more flexible than the dorsal skin, since it must swell after feeding and/or maturation of the gonads. In aquatic species of anuran, however, the dorsal skin must be flexible for decreasing water resistance. In urodeles and apodans, the skin must be more flexible to allow their locomotion.

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